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Atypical signaling of metabotropic glutamate receptor 1 in human melanoma cells☆

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CPCCOEt (PubChem CID: 6278000)
JNJ16259685 (PubChem CID: 11313361)
Forskolin (PubChem CID: 47936)
Dynasore (PubChem CID: 5717066)
Adenosine triphosphate (PubChem CID: 5957)
Isoproterenol (PubChem CID: 3779)
Ionomycin calcium salt (PubChem CID: 6446270)
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ABSTRACT

The metabotropic glutamate 1 (mGlu1) receptor has emerged as a novel target for the treatment of metastatic melanoma and various other cancers. Our laboratory has demonstrated that a selective, non-competitive mGlu1 receptor antagonist slows human melanoma growth *in vitro* and *in vivo*. In this study, we sought to determine if the activation of a canonical G protein-dependent signal transduction cascade, which is often used as an output of mGlu1 receptor activity in neuronal cells, correlated with mGlu1 receptor-mediated melanoma cell viability. Glutamate, the endogenous ligand of mGlu1 receptors, significantly increased melanoma cell viability, but did not stimulate phosphoinositide (PI) hydrolysis in several human melanoma cell lines. In contrast, melanoma cell viability was not increased by quisqualate, a highly potent mGlu1 receptor agonist, or DHPG, a selective group I mGlu receptor agonist. Similarly to glutamate, quisqualate also failed to stimulate PI hydrolysis in mGlu1 receptor-expressing melanoma cells. These results suggest that the canonical G protein-dependent signal transduction cascade is not coupled to mGlu1 receptors in all human melanoma cells. On the other hand, dynamin inhibition selectively decreased viability of mGlu1 receptor-expressing melanoma cells, suggesting that a mechanism requiring internalization may control melanoma cell viability. Taken together, these data demonstrate that the approaches commonly used to study mGlu1 receptor function and signaling in other systems may be inappropriate for studying mGlu1 receptor-mediated melanoma cell viability.

1. Introduction

Melanoma is the most deadly form of skin cancer. In 2014 it is estimated that there will be approximately 75,000 new melanoma cases and nearly 10,000 of these will result in death [1]. One promising approach in melanoma treatment involves inhibiting drivers of oncogenic signaling [2–4]. Despite this progress, many tumors develop resistance to these targeted therapies [5–8] and for a subset of patients there is a lack of treatment options. Therefore, it is critical to identify additional targets to increase the number of treatments available and to delay treatment resistance.

Over half of human melanoma cell lines and biopsies aberrantly express metabotropic glutamate 1 (mGlu1) receptors [9]. Pharmacological inhibition and targeted knockdown of these receptors blocks melanoma growth *in vitro* [10–13] and *in vivo* [10–12], identifying this receptor as a target for melanoma treatment. Early research on the pharmacology and signaling of mGlu receptors was restricted to neuronal cells, leaving gaps in our knowledge of the pharmacology and signaling of mGlu receptors in both non-neuronal and cancer cells [14]. mGlu1 receptors are G protein-coupled receptors activated by the endogenous ligand glutamate. There are eight sub-types of mGlu receptors that are categorized

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based on sequence homology, signal transduction, and pharmacology [15]. In neurons and heterologous expression systems, the canonical signal transduction cascade downstream of mGlu1 receptors is $G\alpha_q$-mediated activation of phospholipase C (PLC) and phosphoinositide (PI) hydrolysis [15–17]. mGlu1 receptors have also been reported to couple to $G\alpha_s$-activated cAMP formation [18] and G protein-independent mechanisms, such as β arrestin-dependent signaling [17,19,20]. Because mGlu1 receptors are emerging as a promising therapeutic target for melanoma, it is important to determine if the pharmacological and signaling properties observed in neurons can be extrapolated to melanoma cells.

Towards this aim, we used the pharmacological profile of mGlu1 receptors determined in heterologous expression systems [17,21–23] and neurons [24] as a starting point. In these systems, our laboratory has shown that the endogenous agonist glutamate, stimulates both PI hydrolysis and increases cell viability [17,21,24]. In contrast, agonists including quisqualate and DHPG were found to only increase PI hydrolysis without affecting cell viability, thereby characterizing them as G protein-biased agonists [17,21,24]. Moreover, these results demonstrate that in these systems PI hydrolysis is not responsible for increases in cell viability.

Here, we report that the signaling of mGlu1 receptors in melanoma cells partially differs from neurons, as neither glutamate nor quisqualate stimulated PI hydrolysis in these cells. In contrast, the inability of quisqualate and DHPG to alter viability in neurons [24], was also observed in mGlu1 receptor-expressing melanoma cells. Furthermore, glutamate increased melanoma cell viability, which was blocked by two selective, non-competitive mGlu1 receptor antagonists. Taken together, these results demonstrate that in melanoma, PI hydrolysis is not responsible for increased cell viability. Since this canonical mGlu1 receptor pathway failed to control melanoma cell viability, we investigated alternative mGlu1 receptor-mediated pathways and found that dynamin-blockade selectively slowed the growth of mGlu1 receptor-expressing melanoma cells. Overall, these findings have important implications for future experimental design when studying mGlu1 receptors in melanoma, and other cancers where mGlu1 receptors have emerged as a therapeutic target [25–27].

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), dialyzed fetal bovine serum, and antibiotic-antimycotic for cell cultures were purchased from Life Technologies (Carlsbad, CA). Glutamate, quisqualate, (S)-3,5-DHPG,7-(Hydroxyimino) cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt), (3,4-dihydro-2H-pyrano[2,3-b]quinolin-7-yl)-(cis-4-methoxycyclohexyl)-methanone (JNJ16259685), forskolin, and dynasore were purchased from Tocris Bioscience (Bristol, United Kingdom). Dimethyl Sulfoxide (DMSO), adenosine 5′-triphosphate magnesium salt, and isoproterenol were purchased from Sigma–Aldrich (St. Louis, MO). Ionomycin calcium salt was purchased from EMD Millipore (Billerica, MA).

2.2. Cell cultures

Five human melanoma cell lines were used in this study. Cells were cultured in 6% CO_2 at 37 °C on 60 mm dishes from MidSci (St. Louis, MO, USA) in DMEM (high glucose) containing 10% FBS, 2 mM glutamine and 2X antibiotic–antimycotic. SK-MEL-2 (SK2) and SK-MEL-5 (SK5) human melanoma cell lines were obtained from Lombardi Comprehensive Cancer Center Tissue Culture Shared Resource (Georgetown University, Washington, D.C., USA). UACC930, C8161, and MV3 melanoma cells were generously provided by Dr. Suzie Chen (Rutgers University, Piscataway, NJ, USA). The following human melanoma cell lines express mGlu1 receptors: SK2 [28,29], SK5 [10,28,29], C8161 [9,11,13,28], and MV3 [9]. UACC930 melanoma cells lack mGlu1 receptor expression [13,28].

2.3. Assessment of cell viability (MTT)

Cells were plated at 2000 cells/well on 96-well plates in a final volume of 100 μl/well. The next day, the medium was replaced with fresh complete medium or medium containing dialyzed serum with or without: ligands (glutamate, quisqualate, DHPG), antagonists (CPCCOEt, JNJ16259685), or dynasore. The selective mGlu1 receptor antagonists (JNJ16259685 and CPCCOEt) and dynasore were dissolved in DMSO, and equal amounts of DMSO were added to controls (1% final). Glutamate and quisqualate were dissolved in equilmolar solutions of NaOH and pH was adjusted to 7.4. DHPG was dissolved in water. After 7 days of treatment, media and drugs were removed and cells were incubated for 40 min at 37 °C with 0.2 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Life Technologies) dissolved in DMEM. Solution was then removed and 70 μl of DMSO were added to dissolve the formazan product, which was measured colorimetrically on a plate reader (Envision, PerkinElmer, Waltham, MA, USA) as described previously [10,17].

2.4. Phosphoinositide (PI) hydrolysis

PI hydrolysis was measured using myo-[3H]inositol and scintillation proximity assay (SPA) beads as described previously [17,21,24]. Cells were plated at approximately 65% confluency and cultured in 96-well plates in 100 μl/well of media containing full serum at 37 °C. After cells reached confluency (3–4 days), cells were heat-shocked (42 °C) for 2.5 h, and then media containing full serum was removed and replaced with media lacking serum and containing 0.625 μCi/well myo-[3H]inositol (PerkinElmer). After an overnight incubation, cells were treated with agonists for one hour at 37 °C in 100 μl/well Locke buffer (156 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO3, 1 mM MgCl2, 1.3 mM CaCl2, 5.6 mM glucose and 20 mM HEPES, pH 7.4) in the presence of 20 mM LiCl to prevent breakdown of inositol phosphates. Treatments were removed, and after lysis in 60 μl/well ice-cold 10 mM formic acid for 30 min, 40 μl/well of cell lysates were transferred to a scintillation plate containing 60 μl/well poly-lysine-coated yttrium SPA beads (PerkinElmer). After 1 hour of vigorous shaking, cell lysates and SPA beads stood at room temperature for 15 h and then inositol phosphates were detected with scintillation counting.

2.5. GloSensor cAMP assay

Relative cAMP formation was measured using the pGloSensor-22F cAMP plasmid and a bioluminescence output as described previously [30]. Cells were plated in a 6-well plate, transfected with pGloSensor-22F cAMP plasmid (Promega, Madison, WI) using lipofectamine LTX and PLUS reagents (Life Technologies), and 24 h later plated in 96-well white-walled plates at 60–80% confluency. The next day, cells were pre-incubated in 1.81 mg/mL of luciferin in a final volume of 100 μl of Locke buffer (Locke-Luc) for 1 h in the dark. Basal bioluminescence was detected every 2 min for a total of 10 min, and then cells were treated with 50 μl of Locke-Luc buffer containing glutamate, forskolin, or isoproterenol (final volume: 100 μl/well). The bioluminescence measured at 12 min post-treatment was divided by the average pre-reads in that well. Responses were normalized to bioluminenscence in the presence of 100 μM forskolin.